APPLICATION FOR UNITED STATES LETTERS PATENT IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

(Case No. 93,509)

Inventors: Susan G. Amara
4100 S.W. 6th Avenue Drive
Portland, Oregon 97201

A Citizen of the United States

Jeffrey L. Arriza
1157 S.W. Florida Street
Portland, Oregon 97219

A Citizen of the United States

Assignee: STATE OF OREGON, Acting by and Through the Oregon State Board of Higher Education on Behalf of the Oregon Health Sciences University, a non-profit organization
3181 S.W. Sam Jackson Park Road
Portland, Oregon 97201-3098

Title: Amino Acid Transporters and Uses

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

AMINO ACID TRANSPORTERS AND USES

~~This invention was made with government support under National Institute of Health grants DA07595 and DA03160. The government has certain rights to this invention.~~

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to amino acid transporters from mammalian species and the genes corresponding to such transporters. Specifically, the invention relates to the isolation, cloning and sequencing of complementary DNA (cDNA) copies of messenger RNA (mRNA) encoding each of four novel human amino acid transporter genes. The invention also relates to the construction of recombinant expression constructs comprising such cDNAs from each of the four novel himan amino acid transporter genes of the invention, said recombinant expression constructs being capable of expressing amino acid transporter proteins in cultures of transformed prokaryotic and eukaryotic cells. Production of the transporter proteins in such cultures is also provided. The invention relates to the use of such cultures of such transformed cells to produce homogeneous compositions of each transporter protein. The invention also provides cultures of such cells producing transporter proteins for the characterization of novel and useful drugs. Antibodies against and epitopes of these transporter proteins are also provided by the invention.

2. Background of the Invention

The approximately 20 naturally-occurring amino acids are the basic building blocks for protein biosynthesis. Certain amino acids, such as glutamate and glycine, as well as amino acid derivatives such as γ-aminobutyric acid (GABA), epinephrine and norepinephrine, and histamine, are also used as signaling molecules in higher organisms such as man. For these reasons, specialized trans-membrane transporter proteins have evolved in all organisms to recover or scavenge extracellular amino acids (*see* Christensen, 1990, Physiol. Rev. 70: 43-77 *for review*).

These transporter proteins play a particularly important role in uptake of extracellular amino acids in the vertebrate brain (*see* Nicholls & Attwell, 1990, TiPS 11: 462-468). Amino

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

acids that function as neurotransmitters must be scavenged from the synaptic cleft between neurons to enable continuous repetitive synaptic transmission. More importantly, it has been found that high extracellular concentrations of certain amino acids (including glutamate and cysteine) can cause neuronal cell death. High extracellular amino acid concentrations are associated with a number of pathological conditions, including ischemia, anoxia and hypoglycemia, as well as chronic illnesses such as Huntington's disease, Parkinson's disease, Alzheimer's disease, epilepsy and amyotrophic lateral sclerosis (ALS; *see* Pines *et al.*, 1992, Nature 360: 464-467).

Glutamate is one example of such an amino acid. Glutamate is an excitatory neurotransmitter (i.e., excitatory neurons use glutamate as a neurotransmitter). When present in excess (> about 300 μM; Bouvier *et al.*, 1992, Nature 360: 471-474; Nicholls & Attwell, *ibid.*; > 5μM for 5 min.; Choi *et al.*, 1987, J. Neurosci. 7: 357-358), extracellular glutamate causes neuronal cell death. Glutamate transporters play a pivotal role in maintaining non-toxic extracellular concentrations of glutamate in the brain. During anoxic conditions (such as occur during ischemia), the amount of extracellular glutamate in the brain rises dramatically. This is in part due to the fact that, under anoxic conditions, glutamate transporters work in reverse, thereby increasing rather than decreasing the amount of extracellular glutamate found in the brain. The resultingly high extracellular concentration of glutamate causes neuron death, with extremely deleterious consequences for motor and other brain functions, resulting in stroke, anoxia and other instances of organic brain dysfunction.

This important role for amino acid transporters in maintaining brain homeostasis of extracellular amino acid concentrations has provided the impetus for the search for and development of compounds to modulate and control transporter function. However, conventional screening methods require the use of animal brain slices in binding assays as a first step. This is suboptimal for a number of reasons, including interference in the binding assay by non-specific binding of heterologous (i.e., non-transporter) cell surface proteins expressed by brain cells in such slices; differential binding by cells other than neuronal cells present in the brain slice, such as glial cells or blood cells; and the possibility that putative drug binding behavior in animal brain cells will differ from the binding behavior in human brain cells in subtle but critical ways. The ability to synthesize human transporter molecules *in vitro* would provide an

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

3

efficient and economical means for rational drug design and rapid screening of potentially useful compounds.

Amino acid transporters are known in the art, and some of these proteins have been isolated biochemically and their corresponding genes have been recently cloned using genetic engineering means.

Christensen *et al.*, 1967, J. Biol. Chem. 242: 5237-5246 report the discovery of a neutral amino acid transporter (termed the ACS transporter) in Erlich ascites tumor cells.

Makowske & Christensen, 1982, J. Biol. Chem. 257: 14635-14638 provide a biochemical characterization of hepatic amino acid transport.

Kanner & Schuldiner, 1987, CRC Crit. Rev. Biochem. 22: 1-38 provide a review of the biochemistry of neurotransmitters.

Olney *et al.*, 1990, Science 248: 596-599 disclose that the amino acid cysteine is a neurotoxin when present in excess extracellularly.

Wallace *et al.*, 1990, J. Bacteriol. 172: 3214-3220 report the cloning and sequencing of a glutamate/aspartate transporter gene termed *gltP* from *Escherichia coli* strain K12.

Kim *et al.*, 1991, Nature 352: 725-728 report the discovery that a cationic amino acid transporter is the cell surface target for infection by ecotropic retroviruses in mice.

Wang *et al.*, 1991, Nature 352: 729-731 report the discovery that a cationic amino acid transporter is the cell surface target for infection by ecotropic retroviruses in mice.

Maenz *et al.*, 1992, J. Biol. Chem. 267: 1510-1516 provide a biochemical characterization of amino acid transport in rabbit jejunal brush border membranes.

Bussolati *et al.*, 1992, J. Biol. Chem. 267: 8330-8335 report that the ASC transporter acts in an electrochemically neutral manner so that sodium ion co-transport occurs without disrupting the normal membrane potential of the cells expressing the transporter.

Engelke *et al.*, 1992, J. Bacteriol. 171: 5551-5560 report the cloning of a dicarboxylate carrier from *Rhizobium meliloti*.

Guastella *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89: 7189-7193 disclose the cloning of a sodium ion and chloride ion-dependent glycine transporter from a glioma cell line that is expressed in the rat forebrain and cerebellum.

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

Kavanaugh *et al.*, 1992, J. Biol. Chem. 267:22007-22009 report that biochemical characterization of a rat brain GABA transporter expressed *in vitro* in *Xenopus laevis* oocytes.

Storck *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89: 10955-10959 disclose the cloning and sequencing of a sodium ion-dependent glutamate/ aspartate transporter from rat brain termed GLAST1.

Bouvier *et al.*, *ibid.*, disclose the biochemical characterization of a glial cell-derived glutamate transporter.

Pines *et al.*, *ibid.*, report the cloning and sequencing of a glial cell glutamate transporter from rat brain termed GLT-1.

Kanai & Hediger, 1992, Nature 360: 467-471 disclose the cloning and sequencing of a sodium ion-dependent, high affinity glutamate transporter from rabbit small intestine termed EAAC1.

Kong *et al.*, 1993, J. Biol. Chem. 268: 1509-1512 report the cloning and sequencing of a sodium-ion dependent neutral amino acid transporter of the A type that is homologous to a sodium-ion dependent glucose transporter.

Nicholls & Attwell, *ibid.*, review the role of amino acids and amino acid transporters in normal and pathological brain functions.

SUMMARY OF THE INVENTION

The present invention relates to the cloning, expression and functional characterization of mammalian amino acid transporter genes. The invention comprises nucleic acids, each nucleic acid having a nucleotide sequence of a novel amino acid transporter gene. The nucleic acids provided by the invention each comprise a complementary DNA (cDNA) copy of the corresponding mRNA transcribed *in vivo* from each of the amino acid transporter genes of the invention. Also provided are the deduced amino acid sequences of each the cognate proteins of the cDNAs provided by the invention.

This invention provides nucleic acids, nucleic acid hybridization probes, recombinant eukaryotic expression constructs capable of expressing the amino acid transporters of the invention in cultures of transformed cells, such cultures of transformed eukaryotic cells that

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

synthesize the amino acid transporters of the invention, homogeneous compositions of each of the amino acid transporter proteins, and antibodies against and epitopes of each of the amino acid transporter proteins of the invention. Methods for characterizing these transporter proteins and methods for using these proteins in the development of agents having pharmacological uses related to these transporter proteins are also provided by the invention.

In a first aspect, the invention provides a nucleic acid having a nucleotide sequence encoding a human neutral amino acid transporter that is the ASCT1 transporter (SEQ ID No:2). In this embodiment of the invention, the nucleotide sequence includes 1680 nucleotides of the human ASCT1 cDNA comprising 1596 nucleotides of coding sequence, 30 nucleotides of 5′ untranslated sequence and 54 nucleotides of 3′ untranslated sequence. In this embodiment of the invention, the nucleotide sequence of the ASCT1 transporter consists essentially of the nucleotide sequence depicted in Figure 1 (SEQ ID No:2). The use of the term "consisting essentially of" herein is meant to encompass the disclosed sequence and includes allelic variations of this nucleotide sequence, either naturally occurring or the product of *in vitro* chemical or genetic modification. Each such variant will be understood to have essentially the same nucleotide sequence as the nucleotide sequence of the corresponding ASCT1 disclosed herein.

The corresponding ASCT1 protein molecule, having the deduced amino acid sequence consisting essentially of the sequence shown in Figure 1 (SEQ ID No.:3), is also claimed as an aspect of the invention. The use of the term "consisting essentially of" herein is as described above. Similarly, the corresponding ASCT1 protein molecule, having the deduced amino acid sequence consisting essentially of the sequence shown in Figure 1 (SEQ ID No.:3), is also claimed as an aspect of the invention. ASCT1 protein molecules provided by the invention are understood to have substantially the same biological properties as the ASCT1 protein molecule encoded by the nucleotide sequence described herein.

In another aspect, the invention comprises a homogeneous composition of the 55.9 kD mammalian ASCT1 transporter or derivative thereof, said size being understood to be the size of the protein before any post-translational modifications thereof. The amino acid sequence of the ASCT1 transporter or derivative thereof preferably consists essentially of the amino acid sequence of the human ASCT1 transporter protein shown in Figure 1 (SEQ ID No:3).

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

In a second aspect, the invention provides a nucleic acid having a nucleotide sequence encoding a human excitatory amino acid transporter that is the EAAT1 transporter (SEQ ID No:4). In this embodiment of the invention, the nucleotide sequence includes 1680 nucleotides of the human EAAT1 cDNA comprising 1626 nucleotides of coding sequence, 30 nucleotides of 5′ untranslated sequence and 24 nucleotides of 3′ untranslated sequence. In this embodiment of the invention, the nucleotide sequence of the EAAT1 transporter consists essentially of the nucleotide sequence depicted in Figure 2 (SEQ ID No:4). The use of the term "consisting essentially of" herein is as described above.

In another aspect, the invention comprises a homogeneous composition of the 59.5 kilodalton (kD) mammalian EAAT1 transporter or derivative thereof, said size being understood to be the size of the protein before any post-translational modifications thereof. The amino acid sequence of the EAAT1 transporter or derivative thereof preferably consists essentially of the amino acid sequence of the human EAAT1 transporter protein shown in Figure 2 (SEQ ID No:5). EAAT1 protein molecules provided by the invention are understood to have substantially the same biological properties as the EAAT1 protein molecule encoded by the nucleotide sequence described herein.

In a third aspect, the invention provides a nucleic acid having a nucleotide sequence encoding a human excitatory amino acid transporter that is the EAAT2 transporter (SEQ ID No:6). In this embodiment of the invention, the nucleotide sequence includes 1800 nucleotides of the human EAAT2 cDNA comprising 1722 nucleotides of coding sequence, 33 nucleotides of 5′ untranslated sequence and 45 nucleotides of 3′ untranslated sequence. In this embodiment of the invention, the nucleotide sequence of the EAAT2 transporter consists essentially of the nucleotide sequence depicted in Figure 3 (SEQ ID No:6). The use of the term "consisting essentially of" herein is as described above.

The corresponding EAAT2 protein molecule, having the deduced amino acid sequence consisting essentially of the sequence shown in Figure 3 (SEQ ID No.:7), is also claimed as an aspect of the invention. EAAT2 protein molecules provided by the invention are understood to have substantially the same biological properties as the EAAT2 protein molecule encoded by the nucleotide sequence described herein.

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

In another aspect, the invention comprises a homogeneous composition of the 62.1 kD mammalian EAAT2 transporter or derivative thereof, said size being understood to be the size of the protein before any post-translational modifications thereof. The amino acid sequence of the EAAT2 transporter or derivative thereof preferably consists essentially of the amino acid sequence of the human EAAT2 transporter protein shown in Figure 3 (SEQ ID No:7).

In yet another aspect, the invention provides a nucleic acid having a nucleotide sequence encoding a human excitatory amino acid transporter that is the EAAT3 transporter (SEQ ID No:8). In this embodiment of the invention, the nucleotide sequence includes 1674 nucleotides of the human EAAT3 cDNA comprising 1575 nucleotides of coding sequence, 15 nucleotides of 5′ untranslated sequence and 84 nucleotides of 3′ untranslated sequence. In this embodiment of the invention, the nucleotide sequence of the EAAT3 transporter consists essentially of the nucleotide sequence depicted in Figure 4 (SEQ ID No:8). The use of the term "consisting essentially of" herein is as described above.

The corresponding EAAT3 protein molecule, having the deduced amino acid sequence consisting essentially of the sequence shown in Figure 4 (SEQ ID No.:9), is also claimed as an aspect of the invention. EAAT3 protein molecules provided by the invention are understood to have substantially the same biological properties as the EAAT3 protein molecule encoded by the nucleotide sequence described herein.

In another aspect, the invention comprises a homogeneous composition of the 57.2 kD mammalian EAAT3 transporter or derivative thereof, said size being understood to be the size of the protein before any post-translational modifications thereof. The amino acid sequence of the EAAT3 transporter or derivative thereof preferably consists essentially of the amino acid sequence of the human EAAT3 transporter protein shown in Figure 4 (SEQ ID No:9).

This invention provides both nucleotide and amino acid probes derived from the sequences herein provided. The invention includes probes isolated from either cDNA or genomic DNA, as well as probes made synthetically with the sequence information derived therefrom. The invention specifically includes but is not limited to oligonucleotide, nick-translated, random primed, or *in vitro* amplified probes made using cDNA or genomic clone embodying the invention, and oligonucleotide and other synthetic probes synthesized chemically

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

8

using the nucleotide sequence information of cDNA or genomic clone embodiments of the invention.

It is a further object of this invention to provide such nucleic acid hybridization probes to determine the pattern, amount and extent of expression of these transporter genes in various tissues of mammals, including humans. It is also an object of the present invention to provide nucleic acid hybridization probes derived from the sequences of the amino acid transporter genes of the invention to be used for the detection and diagnosis of genetic diseases. It is an object of this invention to provide nucleic acid hybridization probes derived from the DNA sequences of the amino acid transporter genes herein disclosed to be used for the detection of novel related receptor genes.

The present invention also includes synthetic peptides made using the nucleotide sequence information comprising the cDNA embodiments of the invention. The invention includes either naturally occurring or synthetic peptides which may be used as antigens for the production of amino acid transporter-specific antibodies, or used for competitors of amino acid transporter molecules for amino acid, agonist, antagonist or drug binding, or to be used for the production of inhibitors of the binding of agonists or antagonists or analogues thereof to such amino acid transporter molecules.

The present invention also provides antibodies against and epitopes of the mammalian amino acid transporter molecules of the invention. It is an object of the present invention to provide antibodies that are immunologically reactive to the amino acid transporters of the invention. It is a particular object to provide monoclonal antibodies against these amino acid transporters, must preferably the human excitatory and neutral amino acid transporters as herein disclosed. Hybridoma cell lines producing such antibodies are also objects of the invention. It is envisioned at such hybridoma cell lines may be produced as the result of fusion between a non-immunoglobulin producing mouse myeloma cell line and spleen cells derived from a mouse immunized with a cell line which expresses antigens or epitopes of an amino acid transporter of the invention. The present invention also provides hybridoma cell lines that produces such antibodies, and can be injected into a living mouse to provide an ascites fluid from the mouse that is comprised of such antibodies. It is a further object of the invention to provide immunologically-active epitopes of the amino acid transporters of the invention. Chimeric

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

antibodies immunologically reactive against the amino acid transporter proteins of the invention are also within the scope of this invention.

The present invention provides recombinant expression constructs comprising a nucleic acid encoding an amino acid transporter of the invention wherein the construct is capable of expressing the encoded amino acid transporter in cultures of cells transformed with the construct. Preferred embodiments of such constructs comprise the human EAAT1 cDNA (SEQ ID No.:4), the human EAAT2 cDNA (SEQ ID No.:6), the human EAAT3 cDNA (SEQ ID No.:8), and human ASCT1 cDNA (SEQ ID No.:2), each construct being capable of expressing the amino acid transporter encoded therein in cells transformed with the construct.

The invention also provides cultures cells transformed with the recombinant expression constructs of the invention, each such cultures being capable of and in fact expressing the amino acid transporter encoded in the transforming construct.

The present invention also includes within its scope protein preparations of prokaryotic and eukaryotic cell membranes containing at least one of the amino acid transporter proteins of the invention, derived from cultures of prokaryotic or eukaryotic cells, respectively, transformed with the recombinant expression constructs of the invention. In a preferred embodiment, each preparation of such cell membranes comprises one species of the amino acid transporter proteins of the invention.

The invention also provides methods for screening compounds for their ability to inhibit, facilitate or modulate the biochemical activity of the amino acid transporter molecules of the invention, for use in the *in vitro* screening of novel agonist and antagonist compounds. In preferred embodiments, cells transformed with a recombinant expression construct of the invention are contacted with such a compound, and the effect of the compound on the transport of the appropriate amino acid is assayed. Additional preferred embodiments comprise quantitative analyses of such effects.

The present invention is also useful for the detection of analogues, agonists or antagonists, known or unknown, of the amino acid transporters of the invention, either naturally occurring or embodied as a drug. In preferred embodiments, such analogues, agonists or antagonists may be detected in blood, saliva, semen, cerebrospinal fluid, plasma, lymph, or any other bodily fluid.

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

10

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the nucleotide (SEQ ID No.:2) and amino acid (SEQ ID No.:3) sequences of the human ASCT1 neutral amino acid transporter.

Figure 2 illustrates the nucleotide (SEQ ID No.:4) and amino acid (SEQ ID No.:5) sequences of the human EAAT1 excitatory amino acid transporter.

Figure 3 illustrates the nucleotide (SEQ ID No.:6) and amino acid (SEQ ID No.:7) sequences of the human EAAT2 excitatory amino acid transporter.

Figure 4 illustrates the nucleotide (SEQ ID No.:8) and amino acid (SEQ ID No.:9) sequences of the human EAAT3 excitatory amino acid transporter.

Figure 5 presents an amino acid sequence comparison between human ASCT1, GLAST1, GLT1 and EAAC1.

Figure 6 illustrates transmembrane electrochemical currents in *Xenopus laevis* oocytes microinjected with RNA encoding ASCT1 and contacted with the indicated amino acids (Panel A); the amino acid concentration dependence of such electrochemical currents (Panel B); and a plot of normalized current *vs.* amino acid concentration illustrating the kinetic parameters of amino acid transport (Panel C).

Figure 7 presents glutamate transporter kinetics of EAAT1 (~~Panel A~~ Panels A and B), EAAT2 (~~Panel B~~ Panels C and D) and EAAT3 (~~Panel C~~ Panels E and F).

Figure 8 represents the pharmacological responsiveness of glutamate transport by the human excitatory amino acid transporters EAAT1, EAAT2 and EAAT3 when contacted with the indicated competitors/ inhibitors at the indicated concentrations.

Figure 9 shows the pattern of expression of EAAT1, EAAT2, EAAT3 and ASCT1 in human tissues; β-actin is shown as a control for amount of RNA in each lane.

Figure 10 shows the pattern of expression of EAAT1, EAAT2, EAAT3 and ASCT1 in human brain tissue; β-actin is shown as a control for the amount of RNA in each lane.

Figure 11 illustrates the degree of predicted amino acid sequence homology between the novel human glutamate transporters EAAT1, EAAT2 and EAAT3; overbars indicate nine

regions of hydrophobicity determined using the algorithm of Eisenberg *et al.*, and potential sites of N-linked glycosylation are shown by the circled asparagine (N) residues.

Figure 12 illustrates electrogenic uptake of various amino acids (Panel A) and the concentration dependence of such uptake of L-glutamate (Panel B) in *Xenopus laevis* oocytes expressing the EAAT1 amino acid transporter.

Table III illustrates Glutamate uptake inhibition constants.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "human amino acid transporter EAAT1" as used herein refers to proteins consisting essentially of, and having substantially the same biological activity as, the protein encoded by the nucleic acid depicted in Figure 2 (SEQ ID No.:4). This definition is intended to encompass natural allelic variations in the EAAT1 sequence. Cloned nucleic acid provided by the present invention may encode EAAT1 protein of any species of origin, including, for example, mouse, rat, rabbit, cat, and human, but preferably the nucleic acid provided by the invention encodes EAAT1 receptors of mammalian, most preferably human, origin.

The term "human amino acid transporter EAAT2" as used herein refers to proteins consisting essentially of, and having substantially the same biological activity as, the protein encoded by the nucleic acid depicted in Figure 3 (SEQ ID No.:6). This definition is intended to encompass natural allelic variations in the EAAT2 sequence. Cloned nucleic acid provided by the present invention may encode EAAT2 protein of any species of origin, including, for example, mouse, rat, rabbit, cat, and human, but preferably the nucleic acid provided by the invention encodes EAAT2 receptors of mammalian, most preferably human, origin.

The term "human amino acid transporter EAAT3" as used herein refers to proteins consisting essentially of, and having substantially the same biological activity as, the protein encoded by the nucleic acid depicted in Figure 4 (SEQ ID No.:8). This definition is intended to encompass natural allelic variations in the EAAT3 sequence. Cloned nucleic acid provided by the present invention may encode EAAT3 protein of any species of origin, including, for example, mouse, rat, rabbit, cat, and human, but preferably the nucleic acid provided by the invention encodes EAAT3 receptors of mammalian, most preferably human, origin.

The term "human amino acid transporter ASCT1" as used herein refers to proteins consisting essentially of, and having substantially the same biological activity as, the protein

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

encoded by the nucleic acid depicted in Figure 1 (SEQ ID No.:2). This definition is intended to encompass natural allelic variations in the ASCT1 sequence. Cloned nucleic acid provided by the present invention may encode ASCT1 protein of any species of origin, including, for example, mouse, rat, rabbit, cat, and human, but preferably the nucleic acid provided by the invention encodes ASCT1 receptors of mammalian, most preferably human, origin.

Each of the nucleic acid hybridization probes provided by the invention comprise DNA or RNA consisting essentially of the nucleotide sequence of one of the amino acid transporters, depicted in Figures 1-4 (SEQ ID Nos.:2,4,6,8), or any portion thereof effective in nucleic acid hybridization. Mixtures of such nucleic acid hybridization probes are also within the scope of this embodiment of the invention. Nucleic acid probes as provided herein are useful for detecting amino acid transporter gene expression in cells and tissues using techniques well-known in the art, including but not limited to Northern blot hybridization, *in situ* hybridization and Southern hybridization to reverse transcriptase - polymerase chain reaction product DNAs. The probes provided by the present invention, including oligonucleotides probes derived therefrom, are useful are also useful for Southern hybridization of mammalian, preferably human, genomic DNA for screening for restriction fragment length polymorphism (RFLP) associated with certain genetic disorders.

The production of proteins such as these amino acid transporter molecules from cloned genes by genetic engineering means is well known in this art. The discussion which follows is accordingly intended as an overview of this field, and is not intended to reflect the full state of the art.

DNA encoding an amino acid transporter may be obtained, in view of the instant disclosure, by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, or by combinations of these procedures, as illustrated below. Screening of mRNA or genomic DNA may be carried out with oligonucleotide probes generated from the nucleic acid sequence information from each of the amino acid transporters disclosed herein. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with know procedures and used in conventional hybridization assays, as described in greater detail in the Examples below. In the alternative, amino acid transporter-

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

B

derived nucleic acid sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, using PCR oligonucleotide primers corresponding to nucleic acid sequence information derived from an amino acid transporter as provided herein. *See* U.S. Patent Nos. 4,683,195 to Mullis *et al.* and 4,683,202 to Mullis.

Each of the amino acid transporter proteins may be synthesized in host cells transformed with a recombinant expression construct comprising a nucleic acid encoding the particular amino acid transporter cDNA. Such recombinant expression constructs can also be comprised of a vector that is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding an amino acid transporter and/or to express DNA encoding an amino acid transporter gene. For the purposes of this invention, a recombinant expression construct is a replicable DNA construct in which a nucleic acid encoding an amino acid transporter is operably linked to suitable control sequences capable of effecting the expression of the amino acid transporter in a suitable host.

The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants. *See*, Sambrook *et al.*, 1990, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press: New York).

Vectors useful for practicing the present invention include plasmids, viruses (including phage), retroviruses, and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous recombination). The vector replicates and functions independently of the host genome, or may, in some instances, integrate into the genome itself. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. A preferred vector is pCMV5 (Andersson *et al.*, 1989, J. Biol. Chem. 264: 8222-8229). Transformed host cells are cells which have been transformed or transfected with recombinant expression constructs made using recombinant DNA techniques and comprising nucleic acid encoding an amino acid transporter protein. Preferred host cells are COS-7 cells

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

(Gluzman, 1981, Cell 23: 175-182). Transformed host cells may express the amino acid transporter protein, but host cells transformed for purposes of cloning or amplifying nucleic acid hybridization probe DNA need not express the transporter. When expressed, each of the amino acid transporters of the invention will typically be located in the host cell membrane. *See*, Sambrook *et al.*, *ibid.*

Cultures of cells derived from multicellular organisms are a desirable host for recombinant amino acid transporter protein synthesis. In principal, any higher eukaryotic cell culture is useful, whether from vertebrate or invertebrate culture. However, mammalian cells are preferred, as illustrated in the Examples. Propagation of such cells in cell culture has become a routine procedure. *See* Tissue Culture, Academic Press, Kruse & Patterson, editors (1973). Examples of useful host cell lines are human 293 cells, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and WI138, BHK, COS-7, CV, and MDCK cell lines. COS-7 cells are preferred.

The invention provides homogeneous compositions of each of the human EAAT1, EAAT2, EAAT3 and ASCT1 amino acid transporter proteins produced by transformed eukaryotic cells as provided herein. Each such homogeneous composition is intended to be comprised of the corresponding amino acid transporter protein that comprises at least 90% of the protein in such a homogenous composition. The invention also provides membrane preparation from cells expressing each of the amino acid transporter proteins as the result of transformation with a recombinant expression construct, as described herein.

Amino acid transporter proteins made from cloned genes in accordance with the present invention may be used for screening amino acid analogues, or agonist or antagonists of amino acid transport, or for determining the amount of such agonists or antagonists in a solution of interest (*e.g.*, blood plasma or serum). For example, host cells may be transformed with a recombinant expression construct of the present invention, an amino acid transporter expressed in those host cells, and the cells or membranes thereof used to screen compounds for their effect on amino acid transport activity. By selection of host cells that do not ordinarily express a particular amino acid transporter, pure preparations of membranes containing the transporter can be obtained.

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

15

The recombinant expression constructs of the present invention are useful in molecular biology to transform cells which do not ordinarily express a particular amino acid transporter to thereafter express this receptor. Such cells are useful as intermediates for making cell membrane preparations useful for transporter activity assays, which are in turn useful for drug screening. The recombinant expression constructs of the present invention may also be useful in gene therapy. Cloned genes of the present invention, or fragments thereof, may also be used in gene therapy carried out homologous recombination or site-directed mutagenesis. *See generally* Thomas & Capecchi, 1987, Cell 51: 503-512; Bertling, 1987, Bioscience Reports 7: 107-112; Smithies *et al.*, 1985, Nature 317: 230-234.

Oligonucleotides of the present invention are useful as diagnostic tools for probing amino acid transporter gene expression in tissues of humans and other animals. For example, tissues are probed *in situ* with oligonucleotide probes carrying detectable groups by conventional autoradiographic techniques, to investigate native expression of this receptor or pathological conditions relating thereto. Further, chromosomes can be probed to investigate the presence or absence of the corresponding amino acid transporter gene, and potential pathological conditions related thereto.

The invention also provides antibodies that are immunologically reactive to the amino acid transporter proteins or epitopes thereof provided by the invention. The antibodies provided by the invention may be raised, using methods well known in the art, in animals by inoculation with cells that express an amino acid transporter or epitopes thereof, cell membranes from such cells, whether crude membrane preparations or membranes purified using methods well known in the art, or purified preparations of proteins, including fusion proteins, particularly fusion proteins comprising epitopes of the amino acid transporter proteins of the invention fused to heterologous proteins and expressed using genetic engineering means in bacterial, yeast or eukaryotic cells, said proteins being isolated from such cells to varying degrees of homogeneity using conventional biochemical means. Synthetic peptides made using established synthetic means *in vitro* and optionally conjugated with heterologous sequences of amino acids, are also encompassed in these methods to produce the antibodies of the invention. Animals that are used for such inoculations include individuals from species comprising cows, sheep, pigs, mice, rats,

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

16

rabbits, hamsters, goats and primates. Preferred animals for inoculation are rodents (including mice, rats, hamsters) and rabbits. The most preferred animal is the mouse.

Cells that can be used for such inoculations, or for any of the other means used in the invention, include any cell line which naturally expresses one of the amino acid transporters provided by the invention, or any cell or cell line that expresses one of the amino acid transporters of the invention, or any epitope thereof, as a result of molecular or genetic engineering, or that has been treated to increase the expression of an endogenous or heterologous amino acid transporter protein by physical, biochemical or genetic means. Preferred cells are *E. coli* and insect SF9 cells, most preferably *E. coli* cells, that have been transformed with a recombinant expression construct of the invention encoding an amino acid transporter protein, and that express the transporter therefrom.

The present invention also provides monoclonal antibodies that are immunologically reactive with an epitope derived from an amino acid transporter of the invention, or fragment thereof, present on the surface of such cells, preferably *E. coli* cells. Such antibodies are made using methods and techniques well known to those of skill in the art. Monoclonal antibodies provided by the present invention are produced by hybridoma cell lines, that are also provided by the invention and that are made by methods well known in the art.

Hybridoma cell lines are made by fusing individual cells of a myeloma cell line with spleen cells derived from animals immunized with cells expressing an amino acid transporter of the invention, as described above. The myeloma cell lines used in the invention include lines derived from myelomas of mice, rats, hamsters, primates and humans. Preferred myeloma cell lines are from mouse, and the most preferred mouse myeloma cell line is P3X63-Ag8.653. The animals from whom spleens are obtained after immunization are rats, mice and hamsters, preferably mice, most preferably Balb/c mice. Spleen cells and myeloma cells are fused using a number of methods well known in the art, including but not limited to incubation with inactivated Sendai virus and incubation in the presence of polyethylene glycol (PEG). The most preferred method for cell fusion is incubation in the presence of a solution of 45% (w/v) PEG-1450. Monoclonal antibodies produced by hybridoma cell lines can be harvested from cell culture supernatant fluids from *in vitro* cell growth; alternatively, hybridoma cells can be

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

injected subcutaneously and/or into the peritoneal cavity of an animal, most preferably a mouse, and the monoclonal antibodies obtained from blood and/or ascites fluid.

Monoclonal antibodies provided by the present invention are also produced by recombinant genetic methods well known to those of skill in the art, and the present invention encompasses antibodies made by such methods that are immunologically reactive with an epitope of an amino acid transporter of the invention. The present invention also encompasses fragments, including but not limited to F(ab) and $F(ab)'_2$ fragments, of such antibody. Fragments are produced by any number of methods, including but not limited to proteolytic cleavage, chemical synthesis or preparation of such fragments by means of genetic engineering technology. The present invention also encompasses single-chain antibodies that are immunologically reactive with an epitope of an amino acid transporter, made by methods known to those of skill in the art.

The present invention also encompasses an epitope of an amino acid transporter of the invention, comprised of sequences and/or a conformation of sequences present in the transporter molecule. This epitope may be naturally occurring, or may be the result of proteolytic cleavage of a transporter molecule and isolation of an epitope-containing peptide or may be obtained by synthesis of an epitope-containing peptide using methods well known to those skilled in the art. The present invention also encompasses epitope peptides produced as a result of genetic engineering technology and synthesized by genetically engineered prokaryotic or eukaryotic cells.

The invention also includes chimeric antibodies, comprised of light chain and heavy chain peptides immunologically reactive to an amino acid transporter-derived epitope. The chimeric antibodies embodied in the present invention include those that are derived from naturally occurring antibodies as well as chimeric antibodies made by means of genetic engineering technology well known to those of skill in the art.

The Examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They set forth for explanatory purposes only, and are not to be taken as limiting the invention.

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

18

EXAMPLE 1

Isolation of a Human Neutral Amino Acid Transporter cDNA

In order to clone a novel human neutral amino acid transporter, a cDNA library was prepared from human motor cortex mRNA using standard techniques [*see* Sambrook *et al.*, 1990, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press: New York)]. Briefly, total RNA was isolated using the method of Chomczynski & Sacchi (1987, Anal. Biochem. 162: 156-159), wherein the tissue is disrupted and solubilized in a solution containing guanidinium isothiocyanate and the RNA purified by phenol/chloroform extractions. Total cellular RNA thus isolated was then enriched for poly (A^+) mRNA by oligo (dT) chromatography. A mixture of oligo (dT)-primed and random-primed mRNA was converted to cDNA using the Superscript Choice System (Bethesda Research Labs, Gaithersburg, MD). cDNA was ligated into the cloning vector λZAPII (Strategene, La Jolla, CA), packaged into phage heads using commercially-available packaging extracts (Strategene) and used to infect *E. coli*. Lawns of infected bacterial cells were used to make plaque lifts for hybridization using standard conditions (*see* Sambrook, *et al.*, *ibid.*).

This cDNA library was hybridized with a ^{32}P-labeled oligonucleotide having the following sequence:

5'-CTG(A/G)GC(A/G)ATGAA(A/G)ATGGCAGCCAGGGC(C/T)TCATACAGGGCTGTGCC-(A/G)TCCATGTT(A/G)ATGGT(A/G)GC-3' (SEQ ID NO:1).

(This oligonucleotide was obtained commercially from Oligos, Etc., Wilsonville, OR). This oligonucleotide was chosen on the basis of shared homology between a cloned rat glutamate transporter gene (GLAST1) and the bacterial glutamate transporter gene *gltP* (*see* Storck *et al*, *ibid.* and Wallace *et al.*, *ibid.*), which suggested an important and conserved structural motif. Hybridization was performed at 50°C in a solution containing 0.5M Na_2HPO_4 (pH 7.15)/ 7% sodium dodecyl sulfate (SDS) and the filters were washed at 60°C in 2X SSPE [0.36M NaCl/ 20 mM sodium phosphate (pH 7.7)/ 2mM ethylenediamine tetraacetic acid (EDTA)] and 1% SDS. Hybridizing clones were identified by autoradiography at -70°C using tungsten-containing intensifying screens (DuPont-NEN, Wilmington, DE).

More than 20 positively-hybridizing clones were detected in screening experiments using the above-described primer. One of these clones was excised from the cloning vector *in vivo*

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

by superinfection with a defective filamentous phage that recognizes and excises cloned insert sequences along with adjacent modified phage replication-form sequences (termed pBluescript SK and available from Strategene). This clone contained a 2.7 kilobase (kb) insert, which was sequenced using the dideoxy-chain termination method of Sanger *et al.* (1977, Proc. Natl. Acad. Sci. USA 74: 5463), using Sequenase 2.0, a modified form of bacteriophage T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, OH). The nucleotide sequence of the portion of this clone containing an open reading frame (encoding the ASCT1 gene) is shown in Figure 1.

This ASCT1 clone (SEQ ID No.:2) was found to be comprised of about 180 bp of 5′ untranslated region, about 900 bp of 3′ untranslated region and an open reading frame of 1596 bp encoding the ASCT1 transporter protein (comprising 532 amino acids). The initiator methionine codon was found to be the first methionine codon 3′ to an in-frame stop codon and embedded within the consensus sequence for eukaryotic translation initiation (*see* Kozak, 1987, Nucleic Acids Res 15: 8125-8132). The ASCT1 amino acid sequence (SEQ ID No.:3; also shown in Figure 1) was found to exhibit similarity to other known glutamate transporter subtypes (an amino acid sequence comparison is shown in Figure 5). An amino acid comparison between glutamate transporters from rat (GLAST1 and GLT-1) and rabbit (EAAC1) showed 39%, 34% and 39% sequence identity (respectively) between these amino acid transporter proteins (shown in Figure 5 by shaded boxes). This degree of sequence identity is comparable to the sequence identity between these glutamate subtypes themselves. Both the amino and carboxyl termini were found to be divergent between these transporter proteins, and diversity was also found in the extracellular domains of these putative protein sequences, which contain conserved potential N-glycosylation sites (shown in Figure 5 by open boxes). It was noted that a highly conserved sequence (comprising the amino acids --LYEA--) in the glutamate transporters was replaced by the unrelated amino acid sequence --IFQC-- in the ASCT1 sequence (at positions 385-387 of the ASCT1 amino acid sequence shown in Figure 5). 6-10 putative transmembrane domains were found using the algorithm of Eisenberg *et al.* (1984, J. Molec. Biol. 179: 125-142). On the basis of these data ASCT1 was determined to encode a related but distinct and novel member of the amino acid transporter family.

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

20

EXAMPLE 2

Isolation of Human Excitatory Amino Acid Transporter cDNA

The remaining (> 20) positively-hybridizing clones from the human motor cortex cDNA library detected by hybridization with the primer described in Example 1 (SEQ ID No.:1) were isolated and the corresponding plasmids obtained by *in vivo* excision after superinfection with defective phage as described in Example 1 above. These resulting plasmids were isolated and purified using conventional techniques (*see* Sambrook *et al.*, *ibid.*). Four classes of clones were distinguished based on differential hybridization experiments using each clone as a hybridization probe against a panel of the remaining clones one after another, where conditions of hybridization stringency were varied to distinguish between each of the classes.

Representative clones from each class were sequenced as described in Example 1. One class of clones represented the ASCT1 cDNA sequences described in Example 1. The other three classes were found to encode novel proteins having amino acid sequences homologous to but distinct from the human ASCT1 sequence. Clone GT5 was determined to contain a 4.0 kb insert encoding a protein having a predicted amino acid sequence (termed EAAT1; SEQ ID No.:4) homologous to but distinct from the rat GLAST1 cDNA clone of Storck *et al.* (*ibid.*). Clone GT13 was determined to contain a 2.5 kb insert comprising an open reading frame corresponding to a full-length coding sequence for a novel human transporter gene termed EAAT2 (SEQ ID No.:6). Clone GT11 was found to contain a partial sequence of another novel human transporter termed EAAT3. The EAAT3 clone was used to re-screen the cDNA library described in Example 1. The result of these re-screening experiments was the isolation of Clone GT11B containing a full-length open reading frame encoding EAAT3 (SEQ ID No.:8).

Figure 11 shows the results of alignment of the predicted amino acid sequences of the three novel glutamate transporters of the invention. Nine regions of Eisenberg algorithm predicted hydrophobicity are denoted by overlining, and potential sites of N-linked glycosylation (consensus sequence N-X-S/T, where X is any amino acid) are indicated by the circles asparagine (N) residues. EAAT1 shares 47% (253/542) amino acid sequence identity with EAAT2 and 46% (262/574) sequence identity with EAAT3, whereas the EAAT2 sequence is 45% (259/574) identical to the predicted EAAT3 sequence. Cross-species comparisons of the predicted amino acid sequences of these novel human glutamate transporters revealed the

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

21

following relationships: EAAT1 was found to be 96% homologous with the rat GLAST1 sequence (Storck *et al.*, *ibid.*); EAAT2 was found to be 90% homologous with the rat GLT1 sequence (Pines *et al.*, 1992, *ibid.*); and EAAT3 was found to be 93% homologous with the rabbit EAAC1 sequence (Kanai & Hediger, 1992, *ibid.*). These results indicate that EAAT1, EAAT2 and EAAT3 are related but distinct members of the glutamate transporter family of amino acid transporters.

EXAMPLE 3

Functional Expression of the ASCT1 Amino Acid Transporter Gene in *Xenopus* Oocytes

The sequence similarity between ASCT1 and the glutamate transporters GLAST1, EAAC1 and GLT-1 suggested that the protein encoded by ASCT1 was an amino acid transporter. The ability of the ASCT1 gene product to transport amino acids, and the identity of which amino acids might be transported by this gene product, was assayed in *Xenopus laevis* oocytes following microinjection of *in vitro* synthesized ASCT1 RNA.

Briefly, the coding sequence of the ASCT1 cDNA was isolated with unique flanking restriction sites using a PCR-based assay. In this assay, each of the complementary primers used for PCR amplification of the coding sequence contained a sequence encoding a unique restriction enzyme recognition site at the 5′ terminus of each PCR primer. For ASCT1, the sense primer contained a *Kpn*I recognition sequence (GGTAC↓C), and the antisense primer contained an *Xba*I recognition sequence (T↓CTAGA) at their respective 5′ termini. Each of the PCR primers used for amplifying ASCT1 sequences had the following sequence:

ASCT1 sense primer:

5′-CGCGGGTACCGCCATGGAGAAGAGCAAC-3′ (SEQ ID NO:10);

ASCT1 antisense primer:

5′-CGCGTCTAGATCACAGAACCGACTCCTTG-3′ (SEQ ID NO:11).

PCR amplification was performed for 30 cycles, each cycle comprising 1 minute at 94°C, 30 seconds at 55°C and 2 minutes at 72°C. Following the PCR, the product of the amplification reaction was purified using standard techniques (Saiki *et al.*, 1988, Science 239: 487-491). The DNA then digested with the restriction enzymes *Kpn*I and *Xba*I and then cloned into the

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

polylinker of an oocyte transcription vector (pOTV; *see* Wang *et al.*, 1991, Nature 352: 729-731) that had been digested with *Kpn*I and *Xba*I. Synthetic RNA was then transcribed *in vitro* from this clone using the method of Kavanaugh *et al.* (1992, J. Biol. Chem. 267: 22007-22009) employing bacteriophage T7 RNA polymerase (New England Biolabs, Beverly, MA). 20-50 nL of ASCT1 RNA (at a concentration of about 400 μg/mL) was injected into defolliculated stage V-VI *Xenopus* oocytes excised from female *Xenopus laevis* anesthetized by immersion in 3-aminobenzoic acid for 60 min. Excised oocytes were treated with collagenase II (Sigma Chemical Co., St. Louis, MO) in calcium-free Barth's saline solution [comprising 88mM NaCl, 1mM KCl, 2.4mM $NaHCO_3$, 0.82mM $MgSO_4$, 7.5mM Tris-HCl (pH 7.6), 50U/mL Nystatin (Sigma) and 0.1mg/mL gentamycin (Sigma)] for 60 min., and then incubated overnight at 15°C in 50% Leibowitz's L-15 media (Grand Island Biological Co. (GIBCO), Long Island, N.Y.). After overnight incubation the oocytes were mechanically defolliculated and then were injected with ASCT-1 RNA and incubated at 19°C for 48h (*see* Kim *et al.*, 1991, Nature 352: 725-728 for further details of *Xenopus* oocyte preparation and microinjection).

Amino acid transport in such oocytes was assayed using [^{3}H] alanine, [^{3}H] serine or [^{35}S] cysteine (obtained from New England Nuclear, Boston, MA). Briefly, microinjected oocytes were patch-clamped at -60mV using a Dagan TEV-200 clamp amplifier with an Axon Instruments (Foster City, CA) TL-1 A/D interface controlled by pCLAMP software (Axon Instruments) (*see* Kavanaugh *et al.*, 1992, J. Biol. Chem. 267: 22007-22009 for a detailed review of this methodology) and continuously superfused with ND-96 buffer (consisting of 96mM NaCl/ 2mM KCl/ 1.8mM $CaCl_2$/ 1mM $MgCl_2$/ 5mM HEPES, pH 7.5). For transport measurements, this solution was changed to a solution containing varying concentrations of the radiolabeled amino acids in ND-96 buffer.

Three types of experiments were performed, the results of each being shown in Figure 6. As shown in Panel A, when such oocytes were contacted with ND-96 buffer containing L-alanine, L-serine or L-cysteine, a hyperpolarization of the cell plasma membrane was produced as the result of inward currents of Na^+ ion, as has been associated with other known amino acid transporters (*see* Nicholls, *ibid.*). In contrast, the amino acids L-lysine, L-glutamine, proline, glycine, methionine, arginine, glutamine, asparagine, and leucine, and the amino acid analogues

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

23

N-methylalanine, had no effect at much higher concentrations (i.e., about 1mM). Another amino acid analogue, 2-methylaminoisobutyric acid (MAIB), which is known to be specific for the amino acid transporter type A (Christensen *et al.*, 1967, J. Biol. Chem. 242: 5237-5246), also had no effect at concentrations of 1mM. Further, in competition experiments, contacting such oocytes with a solution containing MAIB at a concentration of 10mM had no effect on the rate of uptake of [^{3}H] alanine present at 100μM. The response of the oocytes was also stereospecific (D-alanine was found to produce only 12 $\pm$ 3% of the response produced by treatment of these oocytes with L-alanine) and Na^+ ion-specific (no response was detected when Na^+ ions were replaced by *tris*-hydroxyethylaminomethane buffer, shown in Panel A). The rate of radiolabeled amino acid uptake (in pmol/min per oocyte, determined at an amino acid concentration of 100μM) for the amino acids alanine, cysteine and serine are shown in Table I.

The uptake currents measured in ASCT1-injected oocytes were found to be both dose-dependent and saturable. Figure 6, Panel B illustrates the dose-dependency of the electrochemical response of ASCT1-injected oocytes to L-alanine. The intensity of the response (equivalent to the amount of current flow into the cell) increased with the concentration of L-alanine from 10μM to 1mM. The saturability of this response is shown in Figure 6, Panel C. In this Figure, the current, normalized to the maximum response obtained with L-alanine, is shown plotted against the extracellular amino acid concentration of each amino acid tested. For the L-stereoisomers of alanine, serine, cysteine and threonine, the inward current flux was found to saturate and reach a plateau at concentrations from 400-1000 μM. More detailed analyses of the kinetics of amino acid influx were performed by least squares linear regression analysis of induced inward current ([I]) plotted as a function of substrate amino acid concentration ([S]), using the equation shown in the legend of Table II. Data were averaged from all oocytes tested, and the results expressed as the mean $\pm$ standard error are shown in Table II.

These results indicated that the cloned ASCT1 cDNA derived from human motor cortex mRNA encoded an amino acid transporter that was specific for Alanine, Serine, Cysteine (and Threonine) and that amino acid transport activity was accompanied by an inward current flow mediated by sodium ions. These results demonstrated that the novel amino acid transporter isolated herein was related to but distinct from other, known transporters, such as the so-called ASC amino acid transporters (Christensen *et al.*, *ibid.*).

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

24

EXAMPLE 4

Functional Expression of the Glutamate Amino Acid Transporter Genes in *Xenopus* Oocytes

Similar series of experiments were performed using RNA synthesized *in vitro* from constructs containing each of the cloned glutamine transporter genes of the invention. In these experiments, each of the PCR primers used to amplify each of the glutamate transporter genes had the following sequence:

EAAT1 sense primer:

5′-CGCGGGTACCAATATGACTAAAAGCAATG-3′ (SEQ ID NO:12);

EAAT1 antisense primer:

5′-CGCGTCTAGACTACATCTTGGTTTCACTG-3′ (SEQ ID NO:13);

EAAT2 sense primer:

5′-CGCGGGTACCACCATGGCATCTACGGAAG-3′ (SEQ ID NO:14);

EAAT2 antisense primer:

5′-CGCGTCTAGATTATTTCTCACGTTTCCAAG-3′ (SEQ ID NO:15)

EAAT3 sense primer:

5′-CGCGGGTACCGCCATGGGGAAACCGGCG-3′ (SEQ ID NO:16);

EAAT3 antisense primer:

5′-CGCGGGATCCCTAGAACTGTGAGGTCTG-3′ (SEQ ID NO:17).

As can be determined by inspection of these sequences, each of the sense primers contained a *Kpn*I recognition sequence (GGTAC↓C), and each of the antisense primers contained an *Xba*I recognition sequence (T↓CTAGA) at the 5′ terminus of each primer for EAAT1 and EAAT2. For EAAT3, the sense primer contained a *Kpn*I recognition sequence, and the antisense primer contained a *Bam*HI recognition sequence (G↓GATCC) at the 5′ terminus of each primer.

PCR amplification was performed for 30 cycles, each cycle comprising 1 minute at 94°C, 30 seconds at 50°C and 2 minutes at 72°C. Following the PCR, each of the PCR products was isolated and cloned into pOTV as described in Example 3, from which RNA encoding each glutamate transporter was synthesized *in vitro* as described.

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

25

Such RNA preparations were each introduced into *Xenopus* oocytes as described in Example 3 to enable expression therein. Amino acid uptake experiments were performed on such oocytes expressing each of the glutamate transporters, also as described in Example 3. Results of such experiments are shown in Figure 12. Panel A shows electrogenic uptake of various amino acids in EAAT1-expressing oocytes. Both L-glutamate and L-aspartate caused inward currents as high as several microamps when added to the incubation media (ND-96) at a concentration of 100μM. In contrast, incubation of EAAT1-expressing oocytes with L-alanine and L-serine at ten-fold higher concentrations (i.e., 1000μM) did not result in electrogenic uptake of these amino acids. Uptake was found to be stereospecific, since L-glutamate incubation did not result in the generation of an inward electric current, and sodium-ion specific, since electrogenic uptake of L-glutamate was abolished by incubation in sodium ion-free media (choline was used to replace sodium in these incubations).

These experiments also demonstrated the surprising result that cysteine, when present at high enough extracellular concentrations (i.e., 1000μM) was capable of being electrogenically transported by the EAAT1 transporter. Cysteine had not previously been reported to be a glutamate transporter substrate; however, amino acid sequence analysis of the EAAT1 transporter showed structural similarities between EAAT1 and the ASCT1 transporter, which was demonstrated herein to transport cysteine (*see* Example 3). As will be discussed in detail below, the EAAT1 transporter displays a K_m for glutamate of 54μM; in contrast, the K_m for cysteine was found to be 300μM. The EAAT1 transporter thus displays a pattern of substrate specificity that is distinct from that of any known glutamate transporter.

Panel B of Figure 12 illustrates the results of biochemical analysis of substrate affinity of the EAAT1 transporter for glutamate, said results being plotted as current *versus* substrate concentration to yield an estimate of the K_m. These experiments were performed essentially as described for the ASCT1 transporter in Example 3. Patch-clamped oocytes expressing the EAAT1 transporter were incubated with varying extracellular concentrations of L-glutamate, and the magnitude of the resulting inward currents determined. From these experiments, the plotted relationship between the magnitude of the inward current and the extracellular L-glutamate concentration was determined, resulting in an estimate for K_m equal to 54μM for L-glutamate.

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

These results were in good agreement with results obtained in COS-7 cells expressing the EAAT1 transporter, described hereinbelow (*see* Example 5).

EXAMPLE 5

Functional Expression of the Amino Acid Transporter Genes in COS-7 Cells

DNA fragments comprising the coding sequences of the novel glutamate transporter genes of the invention were excised from the pOTV constructs described in Example 3 and subcloned into the mammalian expression plasmid pCMV5 (Anderson *et al.*, 1989, J. Biol. Chem. 264: 8222-8229). These mammalian expression constructs were used for transient expression assays of glutamate transporter protein function after transfection of each of these constructs into COS-7 cells (Gluzman, 1981, Cell 23: 175-182).

Each of the pCMV5 constructs corresponding to EAAT1, EAAT2 and EAAT3 were introduced into COS-7 cells by DEAE-dextran facilitated transfection (*see* Sambrook *et al.*, *ibid.*). Two day following transfection, the transfected cells were washed three times in phosphate-buffered saline (PBS) and then incubated with a mixture of radiolabeled amino acid ([^{3}H]-L-glutamate or [^{3}H]-D-aspartate; Dupont-NEN) and non-radiolabeled amino acid for 10 min. After incubation, the cells were washed three times with ice-cold PBS, solubilized with a solution of 0.1% sodium dodecyl sulfate (SDS) and the amount of radioactivity associated with the cells determined using standard liquid scintillation counting methods. The results of these experiments showed that cells transfected with each of the glutamate transporter constructs accumulated significantly-higher (between 10- and 100-fold higher) amounts of radioactivity than did mock (i.e., pCMV5 plasmid) transfected COS-7 cells (which accumulation represented endogenous COS-7 cell uptake of radioactive glutamate). The course of radioactive glutamate uptake was found to be linear for at least 20 min in assays performed at room temperature.

These results are shown in Figure 7. In the Figure, EAAT1 transporter kinetics of glutamate uptake are depicted in Panel A and of aspartate are shown in Panel B. Similarly, EAAT2 kinetics for glutamate and aspartate are shown in Panels C and D, respectively. Finally, EAAT3 kinetics are shown in Panel E (glutamate) and Panel F (aspartate). Each data point was determined by incubating a COS cell culture transfected with the appropriate pCMV5-glutamate

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

transporter clone with 100nM of radiolabeled amino acid and increasing amounts of unlabeled amino acid. Results are plotted as uptake velocity (in pmol/cell culture/min) minus endogenous uptake *versus* total amino acid concentration, and each data point was performed in triplicate. The results show that both glutamate and aspartate uptake mediated by each of the three novel human glutamate transporters is saturable. Insets in each Panel depict Eadie-Hofstee plots of initial velocity data, from which K_m values were determined. The K_m values are shown as the mean $\pm$ standard error based on at least three independent experiments. These results show that each of the three novel transporter proteins comprising the instant invention is functionally competent as an amino acid transporter when expressed in a culture of mammalian cells, and that each of the novel transporters encoded by the cDNA clones EAAT1, EAAT2 and EAAT3 displays a collection of biochemical properties consistent with their designation as human glutamate transporter proteins.

EXAMPLE 6

Inhibitor Potency Analyses Using COS-7 Cells Expressing Amino Acid Transporter Proteins

COS-7 cell cultures transformed with pCMV5-human glutamate transporter constructs as described in Example 4 were used to characterize the pharmacological properties of each of these transporter proteins relative to a variety of known glutamate transporter inhibitors. These assays were performed essentially as described in Example 4, with the exception that varying amounts of each of a number of known inhibitor compounds were included in the incubations.

The results of these experiments are shown in Figure 8. The data in Figure 8 represent the pharmacological responsiveness of glutamate transport by the human excitatory amino acid transporters EAAT1, EAAT2 and EAAT3 when contacted with the following competitors/ inhibitors: L-*threo*-β-hydroxyaspartate (THA); L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC); L-serine-O-sulfate (SOS); dihydrokainate (DHK); and kainate (KAI). In these experiments, uptake of 1μM of [^{3}H]-L-glutamate was determined in the presence of the indicated amounts of each of the inhibitors. As can be seen from the Figure, each of the glutamate transporter proteins of the invention displays a characteristic pattern of sensitivity to the inhibitors. Thus, the relative

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

28

potency of inhibition of radiolabeled glutamate uptake was found to be as follows for the EAAT1 and EAAT3 transporter proteins:

THA < PDC < SOS << DHK, KAI,

whereas the inhibition pattern for EAAT2 was as follows:

PDC < THA < DHK < KAI < SOS.

These results, as well as results obtained from similar experiments performed with L-cysteate, L-cysteine sulfinic acid, β-glutamate and L-aspartate-β-hydroxymate, are shown in Table III. Even though the relative pattern of inhibition was the same for EAAT1 and EAAT3, the results shown in the Table support the finding that each of the glutamate transporters of the invention is uniquely characterized by its sensitivity to this panel of glutamate uptake inhibitors.

In addition, a number of reported inhibitors were found to be ineffective when tested with COS cell culture expressing each of the novel glutamate transporter proteins of the invention. These include *cis*-1-aminocyclobutane-1,3-dicarboxylate, L-pyroglutamic acid, *S*-sulfo-L-cysteine, *N*-acetyl aspartylglutamate, *N*-methyl-D-aspartate (NMDA) and quisqualate. α-aminoadipate, a classical inhibitor of glutamate uptake, exhibited only low potency when tested against all three EAAT subtypes. These results of functional assays support the conclusion arrived at from structural analysis (i.e., nucleic acid and amino acid sequence analyses) that the glutamate transporter cDNAs and proteins of the invention are novel mammalian transporter species.

EXAMPLE 7

Tissue Distribution of Amino Acid Transporter Expression

The tissue distribution of mRNA corresponding to expression of the amino acid transporters disclosed herein was determined in various tissues by Northern hybridization experiments (*see* Sambrook *et al.*, *ibid.*). The results of these experiments are shown in Figures 9 and 10.

A panel of tissue samples was examined by Northern hybridization analysis performed under high stringency conditions as follows. A nylon filter containing 2 μg human peripheral tissue poly(A)$^+$ RNA was obtained from Clonetech Laboratories (Palo Alto, CA), and a similar filter was prepared containing human brain region RNA as follows. Total RNA was isolated

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

29

from human brain region tissue obtained from the Oregon Brain Repository and 20μg/ region were size-fractionated by denaturing formaldehyde agarose gel electrophoresis (*see* Sambrook *et al.*, *ibid.*). Fractionated RNA was then transferred to a nylon filter using the Northern blot/ capillary-osmotic technique. Northern hybridization of both filters was performed individually with ^{32}P-labeled amino acid transporter-specific probes for each transporter to be analyzed. Probes were derived from amino acid transporter coding sequences and labeled using ^{32}P-labeled dCTP by the random primer method (Boehringer-Mannheim, Indianapolis IN). Filters were hybridized overnight at 42°C individually with each radiolabeled probe (at a concentration of 10^6 cpm/mL) in a solution of 5X SSPE/ 50% formamide/ 7.5X Denhardt's solution (comprising 0.15g/100mL each of Ficoll, polyvinylpyrrolidone and bovine serum albumin)/ 2% SDS and 100μg/mL denatured salmon-sperm DNA. Following hybridization, filters were washed twice for 30 min at room temperature in 2X SSPE/ 0.1% SDS and twice for 20 min at 50°C in 0.1X SSPE/ 0.1% SDS. Hybridizing RNAs were visualized by autoradiography at -70°C using intensifying screens. The filters were subsequently re-probed as described with a radiolabeled human β-actin probe (Clonetech) as a positive control.

The results of these experiments are shown in Figures 9 and 10. Figure 9 illustrates expression of each of the amino acid transporters in human heart, brain, placenta, lung, liver, muscle, kidney and pancreas. The size (in kb) of the transcripts corresponding to expression of each transporter are displayed along the right-hand border of each panel. As is seen from these autoradiographs, EAAT1 is expressed predominantly in brain, heart and muscle, to a lesser extent in placenta and lung, weakly in liver, and at levels below the ability of this assay to detect in kidney and the pancreas (if at all). EAAT2 is expressed in brain, and to a lesser extent in placenta; expression was not detected in any other tissue tested. EAAT3 is expressed predominantly in the kidney, but significant expression was also detected in brain, placenta, and lung. ASCT1 is expressed in all tissues tested as at least one of three differently-sized transcripts, possibly corresponding to differential RNA processing during expression of this transporter (which result might be due in the alternative to the utilization of alternative polyadenylation sites found in the 3′ untranslated region). These results demonstrate that the amino acid transporters disclosed herein are encoded by separate and distinct, albeit related, genes and that each transporter has a unique pattern of tissue-specific expression.

30

Figure 10 shows the distribution of these amino acid transporter transcripts in different human brain regions. Varying expression levels were found for each of the amino acid transporters in all brain regions examined. These results support the conclusion that the amino acid transporters of the invention may play an important role in normal brain function, and that disruption of amino acid transport by these transporter may be important determinants in organic brain dysfunction, as a result of ischemia or anoxia.

EXAMPLE 8

Construction of Vaccinia Virus-Recombinant Expression Constructs for Functional Expression of Amino Acid Transporters

Using an alternative approach, the amino acid transporter proteins of the invention are expressed in human HeLa (vulval adenocarcinoma) cells *via* a vaccinia virus-based construct. In these experiments, each of the amino acid transporter cDNAs of the invention are excised from their respective pOTV-containing constructs and subcloned into a modified pBluescript (Strategene) vector wherein each of the amino acid transporter cDNAs described above is under the control of a bacteriophage T7 RNA polymerase promoter (as is described in Blakely *et al.*, 1991, Anal. Biochem. 194: 302-308), termed pT7-AAT constructs. HeLa cells are first infected with a recombinant vaccinia virus, VTF-7, that expresses T7 RNA polymerase. Cells are incubated with virus at a concentration of about 10 plaque-forming unit/cell in serum-free Dulbecco's modified Eagle's medium at 37°C for 30 min., and then the cells were transfected with each of the amino acid transporter constructs described above (i.e. the pT7-AAT constructs) using a lipofectin-mediated (Bethesda Research Labs, Gaithersburg, MD) transfection protocol (*see* Felgner *et al.*, 1987, Proc. Natl. Acad. Sci. USA 84: 7413-7417). Cells are then incubated for 12-24h before being assayed for amino acid transporter expression as described in Example 5.

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

31

EXAMPLE 9

Construction of Fusion Proteins-Recombinant Expression Constructs for Expression of Immunologically-Active Epitopes of Amino Acid Transporters

The amino acid transporter proteins of the invention are expressed as fusion proteins in bacteria to produce immunologically-active epitopes. In these experiments, each of the amino acid transporter cDNAs of the invention are excised from their respective pOTV-containing constructs and subcloned into a pGEX-2T construct (Pharmacia, Piscataway, N.J.) whereby the coding sequences of the amino acid transporter cDNAs are translationally in-frame with sequences encoding glutathione-*S*-transferase (described in Arriza *et al.*, 1992, J. Neurosci. 12: 4045-4055), termed pGST-AAT constructs. After introduction of the pGST-AAT constructs into bacterial cells (*E. coli*, strain D5α) using conventional techniques (*see* Sambrook *et al.*, *ibid.*), fusion protein expression is induced with isopropyl-1-thio-β-D-galactopyranoside as described (Smith & Johnson, 1988, Gene 67: 31-40) and are purified using glutathione-Sepharose 4B (Pharmacia). Antibodies are then raised against each of the amino acid transporters of the invention by inoculation of rabbits with 300-500 μg of purified fusion protein in Freund's adjuvant (Grand Island Biological Co., Grand Island, NY), said inoculation repeated approximately every 4 weeks. Sera are immunoaffinity-purified on columns of Affi-Gel 15 derivatized with purified fusion protein. After salt elution, such antibodies are neutralized, stabilized with bovine serum albumin at a final concentration of 1mg/mL, dialyzed against PBS and assayed by immunoblotting using conventional techniques (Harlow & Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

TABLE I

Amino Acid (1mM)*	ASCT1 RNA-injected Oocytes**	Water-injected Oocytes**
Alanine	18 ± 2	0.6 ± 0.1
Serine	20 ± 5.1	0.4 ± 0.1
Cysteine	19.2 ± 5.9	1.0 ± 0.3

* n = 5; ** pmol/min per oocyte:

TABLE II

Amino Acid*	K_m (μM)	I_{max}**
Alanine	71 ± 14	(1.0)
Serine	88 ± 11	1.2 ± 0.08
Cysteine	29 ± 6	1.0 ± 0.04
Threonine	137 ± 19	1.4 ± 0.03
Valine	390 ± 8	0.6 ± 0.11

NOTE: data is expressed as the mean of at least 5 determinations ± standard error.

* All amino acids were the L-stereoisomer

** I_{max} was determined by least squares fit to the equation:

$$I = I_{max} \times ([S]/(K_m + [S])$$

where I_{max} is the maximal current and K_m is the transport constant

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000

Glutamate uptake inhibition constants.

Compound	Ki (in μM) determined for each transporter[a]		
	EAAT1	EAAT2	EAAT3
THA (L-threo-β-hydroxyaspartate)	32±8	19±6	25±5
PDC (L-trans-pyrrolidine-2,4-dicarboxylate)	79±7	8±2	61±14
SOS (L-Serine-O-sulfate)	107±8	1157±275	150±52
DHK (Dihydrokainate)	> 1 mM	23±6	> 1 mM
KAI (Kainate)	> 1 mM	59±18	> 1 mM
L-cysteate	10±3	10±2	19±9
L-cysteine sulfinic acid	14±7	6±1	17±2
β-glutamate	297±118	156±37	307±48
L-aspartate-β-hydroxymate	369±70	184±27	133±34

[a] Under the assay conditions used ([S]<<Km), the Ki value does not differ significantly from the measured IC50.

Table III

ALLEGRETTI & WITCOFF, LTD.
10 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 715-1000